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Differences in metabolite levels upon differentiation of intact neuroblastoma × glioma cells observed by proton NMR spectroscopy

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Proton NMR spectroscopy was used to study the effect of differentiation with prostaglandin E_1 and theophylline on intact hybrid neuroblastoma × glioma cells. The standard proton NMR method showed more resolvable signals than the spin echo NMR spectra. The differentiated cells were found to contain significantly higher levels of glutamine than the undifferentiated precursors. Observations on cell extracts confirmed these results.

Differentiation *Neuroblastoma × glioma cell* *Proton NMR* vs *spin-echo* *Glutamine* *Theophylline* *Prostaglandin E_1*

1. INTRODUCTION

Two approaches are taken in applying NMR to study cell metabolism: either the method is applied non-invasively to intact cells; and/or the cells are treated to produce extracts [1]. In the latter case a series of extracts obtained sequentially in time provide a measure of metabolic changes, and this approach has the advantage that in extracts, as opposed to cells, the NMR signals of all components are sharp, easily quantitated and identifed. In cells, becasue of local field inhomogeneities and viscosity effects, the signals tend to be broadened, and additionally some components which are immobilized by binding to membrane or protein may not be observed if their linewidths are too large. Nevertheless, we feel that direct non-invasive observation of a metabolic process in intact cells is the preferable approach, particularly since in the extraction process some hydrolysis of acid- or base-sensitive components can occur, as found in this work.

We report here the observation of differences in glutamine concentrations in intact hybrid neuroblastoma × glioma cells, which differ in whether they are undifferentiated or differentiated following treatment with prostaglandin E_1 and theophylline. We have found that standard proton NMR observation provided more information in this case than the spin echo NMR method [2].

2. EXPERIMENTAL

NG108-15 cells, passage 15–17 of a clonal line derived by fusion of a rat C6 glioma clone and a mouse N18 neuroblastoma clone (T. Amano, B. Hamprecht and M. Nirenberg, unpublished), were grown for 5–7 days in 90% Dulbecco's Modified Eagle Medium (Gibco 430-2100): 10% fetal bovine serum, containing hypoxanthine (100 μM) and thymidine (16 μM). In parallel cultures, a separate set of cells were grown under the same conditions in the presence of theophylline (1 mM) and pro-

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staglandin E_1 (2–5 μM) (Upjohn). In the presence of these agents the cells were induced to differentiate as evidenced by markedly enhanced neurite extension. The undifferentiated cells were begun at 5–10% confluence and the differentiated cells at 10–20% confluence, and both were harvested at

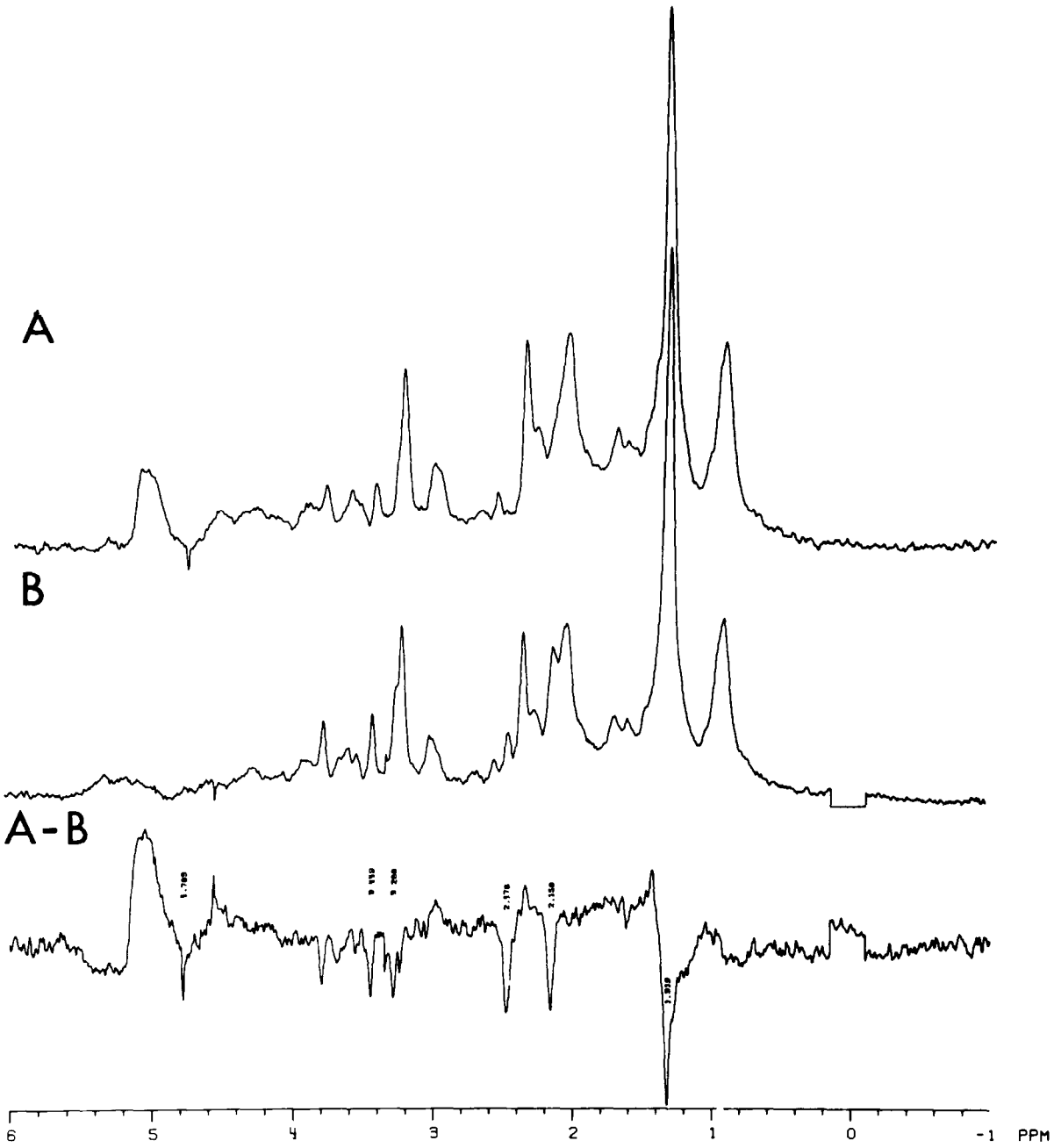

Fig.1. Proton NMR spectra at 500 MHz of undifferentiated hybrid neuroblastoma × glioma cells (A), differentiated cells (B) and the difference between them. The spectra were run under the same spectroscopic conditions and were normalized using the absolute intensity (AI) command. The peaks at 4.5–5.2 ppm result from water suppression, the other peaks are discussed in the text.

~80% confluence. The growth medium for preparation of cell extracts was replaced with fresh medium 4 h before the experiment to avoid depletion of any components.

NMR spectra were recorded at 500 MHz on a Nicolet, Inc. NT500 spectrometer at 20 ± 1°C. Most spectra were recorded in 20% D_2O to provide a lock signal, and the H_2O peak was suppressed using a DANTE sequence [3,4] with a low power selective pulse (5 μs) in a nested loop (128 cycles) followed by a high power pulse (10 μs); 16 scans were collected with 4000 data points. Spectra of cells were obtained on suspensions of ~10^8 cells/ml, which were washed with phosphate buffer (50 mM) containing KCl and NaCl (10 mM each). The cells were allowed to settle under gravity for ~10 min, to ~1/2 in. packed cells. At the end of the experiment the cell supernatant was tested and showed no significant proton-containing components other than water. ^{31}P spectra were obtained at 109.3 MHz on the NIH 270 spectrometer with a 700 ms recycle time and 500–1000 scans in 4000 data points. Extracts were made by treatment with cold 35% perchloric acid to a final concentration of 10% as in [1], except for neutralization with KOH solution.

3. RESULTS AND DISCUSSION

Proton NMR spectra of suspensions of differentiated and undifferentiated hybrid neuroblastoma × glioma cells showed significant differences in the aliphatic region (fig.1). These spectra were normalized to the same intensity scale before the difference spectrum was taken. By comparison with known spectra of amino acids it was found that the difference peaks at 2.16, 2.48 and 3.79 ppm corresponded to glutamine, and not to glutamic acid (fig.2). Other difference peaks were observed, but apart from the assignment of that at 1.3 ppm to methyl groups, no specific origin could be assigned to these peaks. The differences at ~4.5–5.2 ppm derive from water suppression. That these cells were still metabolically active under these conditions was shown by the retention of ATP signals in the ^{31}P NMR spectra (fig.3) for up to 30 min after packing the cells.

To check these observations on the intact cells, extracts were made, and proton NMR spectra were reproducibly found to have a similar difference in

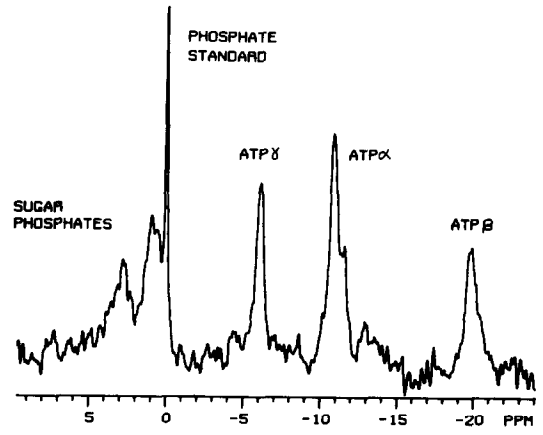

Fig.2. Proton NMR spectra of glutamine (pH 8.2) and glutamic acid (pH 7.75). The chemical shifts of the upfield peaks are Gln, 2.45, 2.14 ppm; Glu, 2.35, 2.14 and 2.06 ppm.

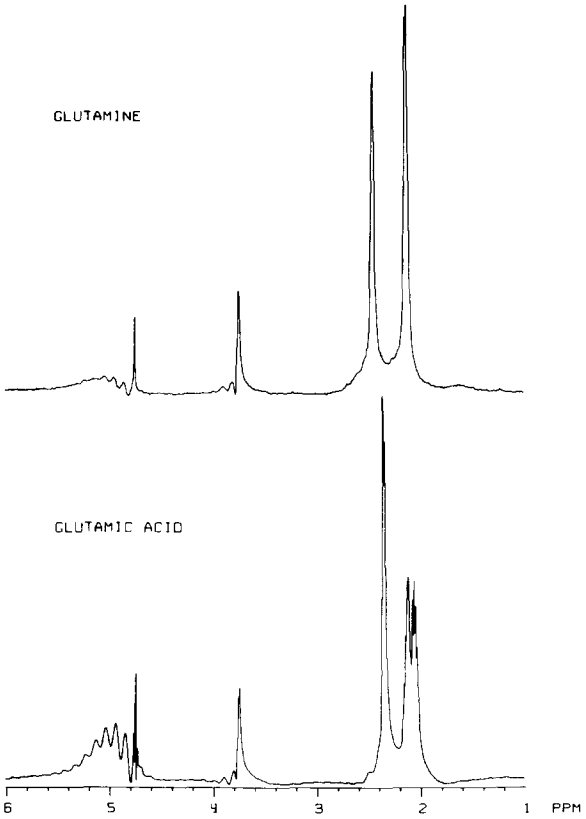

Fig.3. ^{31}P NMR spectrum at 109.3 MHz of packed undifferentiated NG108 cells.

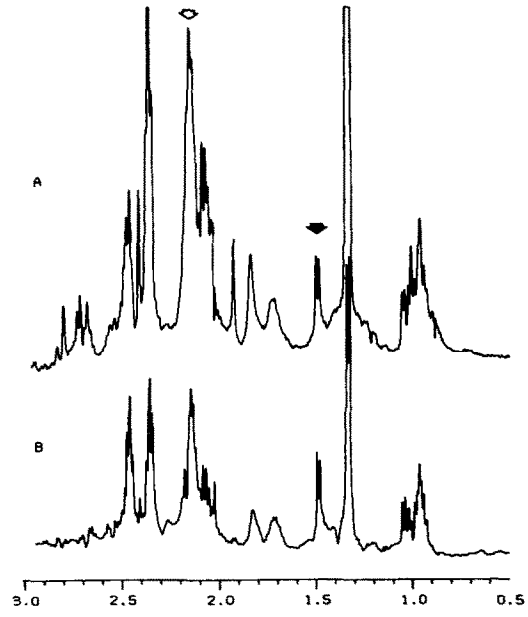

Fig.4. Proton NMR spectra of extracts of differentiated (A) and undifferentiated (B) cells. The doublet at 1.48 ppm (filled arrows) which derives from Ala was used for normalization. There was an approximate 3-fold increase in the relative intensity of the peak at 2.14 ppm (open arrow) in A, corresponding to an equivalent increase in the Gln plus Glu levels. The major doublet at 1.32 ppm corresponds to lactic acid.

glutamine/glutamic acid levels (fig.4). It should be noted that in these spectra there were differences in the intensities of peaks attributable to Glu, particularly that at 2.06 ppm. However, since no such difference was observed in the intact cells this was attributed to hydrolysis due to the extraction procedure, and thus the total of Glu plus Gln peaks (represented by the common peak at 2.14 ppm) was used to estimate the ratio of Gln concentrations in the two cases. These results are not due to depletion of glutamine in the medium [5] but are consistent with induction of glutamine synthetase upon differentiation [6].

Spin echo spectra of these cells showed much less detail and the differences due to glutamine were not observed (fig.5). In this case the advantages claimed for spin-echo *vs* standard proton NMR observation [2] were not realized. The reasons for this are not obvious however, since the spin-echo method should give signals from relatively mobile components, while the signals as have been observed in standard proton spectra

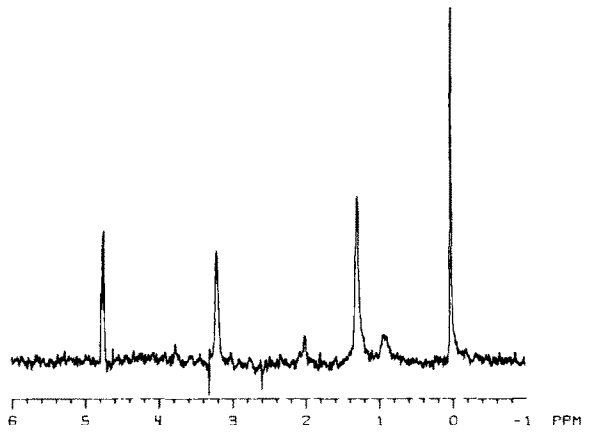

Fig.5. Spin-echo proton NMR spectra of differentiated neuroblastoma × glioma cells. The delay between the 90° and 180° pulses in this spectrum was 30 ms.

(fig.1) are quite sharp, also indicating a fairly high degree of mobility. One possible reason for the absence of the sharp signals in the spin-echo spectra might include partial immobilization of the Gln residues by binding or exchange between sites, so that the delay (τ) used between pulses in the spin-echo pulse sequence excludes them. The signals represented by these Gln residues might also derive from some derivatized form, such as a peptide, although this seems unlikely since only a few other resonances which could derive from other residues are observed in the difference spectrum.

ACKNOWLEDGEMENTS

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